and other provitamins A directly to a hypothetical compound having vitamin A activity without a recognizable absorption spectrum or reaction with antimony trichloride.

It was hoped that a study of this species both as aquatic larvae and as terrestrial adults would prove a fair test of Wald's (1946) hypothesis, particularly since Wald (1947) recorded that 'such a permanently larval salamander as the mudpuppy (*Necturus maculosus*) contains only vitamin(s) A₂ in its retina as an adult'. As no vitamin A was found in axolotls, the original aim was defeated, but new problems have arisen out of the apparent dispensability of the vitamin for this species.

SUMMARY

1. The purpose of the present work was to investigate the proportions of vitamins A₁ and A₂ in an animal (*Amblystoma tigrinum*) which can exist both as sexually mature aquatic larvae and as terrestrial adults.

2. The vitamin A and carotenoid contents of the livers of *A. tigrinum* on various diets have been determined.

3. No conversion of β -carotene to vitamin A could be observed. Unless preformed vitamin A had been added to the diet, no vitamin A could be detected in the livers.

4. It is concluded that the requirements of this species for vitamin A must be either extremely small, or non-existent, or, alternatively, that an unrecognized compound other than vitamin A₁ is present and can replace the vitamin in physiological processes.

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Studies in Vitamin A

24. SPECTROSCOPIC EXAMINATION OF THE LIPIDS OF TWO SPECIES OF NEWTS

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In a previous paper (Collins, Love & Morton, 1953a) the history of the discovery of vitamin A₂ in amphibians is briefly recounted. So far, the only amphibians which have been found to contain vitamin A₂ are the newt, *Triturus viridescens* (Wald, 1942), and the bullfrog tadpole, *Rana catesbiana* (Wald, 1946). It is the purpose of the present paper to report on an examination of two species of newts, *Triturus cristata* and *T. carnifex*, for the presence of vitamins A₁ and A₂.

EXPERIMENTAL

Animals. Great newts (*T. cristata*) and Italian great newts (*T. carnifex*) were bought from a dealer. Some newt larvae (*T. cristata* and *T. vulgaris*) were caught near Speke, Liverpool.

Extraction. The tissues were ground up with Na₂SO₄ (anhydrous) and silver sand and extracted with light petroleum (b.p. 40–60°).

Chromatography. Alumina (P. Spence and Co., Widnes, grade O) was weakened by adding 10% (w/w) of water. This material was used for all chromatographic separations in conjunction with light petroleum (b.p. 40–60°) or diethyl ether or mixtures of the two solvents.

Estimation of vitamin A. This was accomplished either from the ultraviolet absorption using the data given by Cama, Collins & Morton (1951) or from the SbCl₃ colour test. In the latter case the absorptions at 620 m μ . ($E_{620}^{1\%}$) and at 693 m μ . ($E_{693}^{1\%}$) were measured in a visual spectrophotometer (Hilger-Nutting). In order to calculate the amounts of vitamin A₁ and vitamin A₂, use was made of the data given by Shantz (1948) and by Cama *et al.* (1951). For the pure substances, $E_{1\text{cm}}^{1\%}$ vitamin A₁ at 620 m μ . = 5070, $E_{1\text{cm}}^{1\%}$ vitamin A₂ at 693 m μ . = 4100. As vitamin A₂ absorbs at 620 m μ . the

following correction has to be made: extinction at 693 $m\mu$. due to vitamin $A_2 = E_{693 m\mu}$; extinction at 620 $m\mu$. due to vitamin $A_1 = E_{620 m\mu} - 0.308 \times E_{693 m\mu}$.

Saponification. The extracted lipids were mixed with 60% (w/v) KOH and ethanol in the proportions 1:2:5. The mixture was left in the dark at room temperature until the solution was homogeneous (1-2 days). The unsaponifiable material was then extracted with diethyl ether. Although saponification may not have been quite complete it was advantageous not to heat strongly because of the risk of isomerizing carotenoids and producing 'cis' peaks in the absorption spectra.

Phase separation. The unsaponifiable material was dissolved in light petroleum (b.p. 40-60°) and shaken with methanol containing 10% (v/v) water. Eight successive portions of aqueous methanol were used to extract the light petroleum.

RESULTS

Preliminary experiments (using *T. cristata*). The unsaponifiable material from the livers of eighty great newts was chromatographed. Both vitamin A_1 and vitamin A_2 were present. An attempt was made to extract the visual pigment from the eyes, the aim being to see whether both rhodopsin and porphyropsin were present. The amount obtained was too small to establish unequivocally the presence of either.

***T. carnifex*.** This species was used in the subsequent work because it was easier to obtain a sufficient number of animals. A full investigation was made of the lipids from 140 newts. The oil from each organ was saponified in the cold and a phase separation was done on the unsaponifiable material. Table 1 shows the weights of tissue and of lipid, and the percentage of lipid. The absorption spectra of the lipids are shown in Fig. 1. The distribution of

carotenoids between 'carotenes' and 'xanthophylls' was determined by a phase separation. The peak value of $E_{1 cm}^{1\%}$ for the individual carotenoids was taken to be 2500, and the amounts of carotenoids so calculated are shown in Table 2.

Table 1. *Lipid content of the organs from 140 newts (Triturus carnifex)*

Organ	Fresh wt. (g.)	Wt. of lipid (g.)	Lipid in tissue (%)
Livers	34.7	0.862	2.49
Fat bodies (male)	1.83	1.135	62.2
Fat bodies (female)	1.38	0.7665	55.6
Ovaries	7.10	0.187	2.63
Testes	4.72	0.119	2.53
Spleens	2.44	0.010	0.42
Alimentary tracts	79.49	2.587	3.26
Carcasses*	(73.00)	(0.702)	0.96

* A sample only.

Table 2. *Carotenoid content of newt organs*

(The unsaponifiable material was partitioned between light petroleum and methanol as described in the text. The value of $E_{1 cm}^{1\%}$ (448 $m\mu$, light petroleum) has been assumed to be 2500.)

Organ	Epiphasic (hydrocarbons) (mg./100 g. lipid)	Hypophasic (hydroxylated carotenoids) (mg./100 g. lipid)
Livers	237	139
Fat bodies (male)	3.2	6.6
Ovaries	24	28
Testes	15	24
Spleens	26	33
Alimentary tracts	3.4	3.4
Carcasses*	27	79

* A sample only, including the highly pigmented skin.

Fig. 1. Absorption spectra of the whole lipids from the organs of *T. carnifex*. The solvent was light petroleum (b.p. 40-60°) and the values of $E_{1 cm}^{1\%}$ are calculated back to the equivalent of 1 g. of tissue/100 ml., using a 1 cm. cell. ----, testes ($\times 10$); —, livers; - · - · -, alimentary tract ($\times 10$); · · · · ·, fat bodies (female); · · · · ·, spleens ($\times 10$); —, ovaries ($\times 5$); · · · · ·, carcasses ($\times 10$); —○—○—, fat bodies (male). (In this diagram the actual intensities of absorption have been multiplied in those cases where a factor is given in brackets.)

The liver oil was examined in greater detail by means of chromatography. The results are shown in Fig. 2, for the portion of the unsaponifiable fraction which partitioned into the methanol phase. The presence of vitamin A₂ is shown by the colour test peak at 693 m μ ., the displacement in the direction of longer wavelengths of the vitamin A₁ peak at

other selectively absorbing substances which have not been identified, but which may include steroids.

Some newt larvae (mixed *T. cristata* and *T. vulgaris*) were examined for vitamin A; forty-three newts, weighing 13.7 g., yielded an unsaponifiable fraction showing an inflexion at 335 m μ . On chromatography a fraction was obtained with a

Fig. 2. Absorption spectra of the fractions obtained on chromatography of the liver unsaponifiable material from *T. cristata*. The solvent was light petroleum (b.p. 40–60°) and the values of $E_{1\%}^{1\text{cm}}$ are calculated back to the equivalent of 1 g. liver/100 ml., 1 cm. cell. Fraction: 1, —, adsorbed weakly; 2, ----, ($\times 0.5$); 3, - · - · - ·; 4, · · · · ·; 5, · · · · ·; 6, ○—○—○, adsorbed strongly.

Table 3. Vitamins A₁ and A₂ in some organs of a newt (*Triturus cristatus*)

Organ	Vitamin A ₁ (mg./100 g. lipid)	Vitamin A ₂ (mg./100 g. lipid)
Liver: fractions obtained by chromatography:		
(1) adsorbed weakly	7.9	None detected
(2)	35.4	50.6
(3)	9.0	20.1
(4)	3.8	7.7
(5) adsorbed strongly	3.3	4.6
Total	59.4	83.0
Ovary	1.7	4.6
	($\mu\text{g./eye}$)	($\mu\text{g./eye}$)
Eye	0.030	0.039

328 m μ . and the presence of the subsidiary ultra-violet maximum at about 286 m μ . The amounts of vitamins A₁ and A₂ in various tissues and lipid fractions are shown in Table 3. The ultraviolet absorption curves also indicate the presence of

maximum in the absorption spectrum at 333–335 m μ . With SbCl₃ a transitory greenish blue colour developed but was too weak for the position of the maximum to be seen in the visual spectrophotometer. However, there is here very strong presumptive evidence for the presence of vitamin A₂.

DISCUSSION

The amount of lipid in the various tissues was normal (1–4%) except for the fat bodies which, as was to be expected, were rich in lipid (50–60%). All the tissues contained carotenoid. The lipids from the various tissues all exhibited some selective absorption between 250 and 300 m μ . The characteristic absorption maxima at 293, 281, 271 m μ . of 7-dehydrosteroids (steroid 5:7-dienes) were recorded for some tissue lipid fractions. In some tissue extracts (e.g. ovaries, male fat bodies and carcasses) the 7-dehydrosteroid absorption is superimposed on obvious unselective absorption and by

drawing freehand a continuation of the slowly rising absorption on the long-wave side of 300 m μ . and the more steeply rising absorption below 270 m μ ., the end absorption between 270 and 300 m μ . can be interpolated (cf. Glover, Glover & Morton, 1952). If this approximation is subtracted from the original spectrum, the extinction due to 7-dehydrosteroid can be obtained. Making this correction the amounts of 7-dehydrosteroids calculated from the corrected absorption at 282 m μ . were 15.5 mg./100 g. lipid for the ovary, 0.42 mg./100 g. lipid for the male fat body, 21.8 mg./100 g. lipid for the carcass and 0.39 mg./100 g. lipid for the liver (from the results of chromatography). The carcass included the skin and the relatively high proportion of 7-dehydrosteroid is not out of line with experience on other animals in which skin 7-dehydrosteroid is the presumed source of vitamin D. As 7-dehydrosteroids were not especially looked for it is not possible to say if they were present in all tissues, but qualitative evidence for their presence was obtained with female fat bodies and the alimentary tract.

The presence of vitamin A₂ was established beyond doubt. In all the organs where enough vitamin was present for quantitative analysis there was more vitamin A₂ than vitamin A₁. Wald's (1947) observations on *T. viridescens* were made on eyes only. This animal passes its early life in fresh-water as a gilled larva and after several months metamorphoses to the land-living red eft. At this

stage both A₁ and A₂ are found in the eyes, A₁ predominating. After 1-2 years of growth the animal undergoes a second metamorphosis and returns to water for the rest of its life. At this stage vitamin A₂ preponderates over A₁, although both vitamins are present. There is a clear need to study the distribution of vitamins A₁ and A₂ in various tissues of this species at different stages in its life history. Wald's observations are consistent with the idea that during its period as a land animal the red eft is not obtaining dietary vitamin A₂ or synthesizing it. The larva may have acquired a store of A₂ which is not augmented until the final return to water.

The present observations confirm, for the two species studied, the preponderance of vitamin A₂ over vitamin A₁ in the main storage depots. The bearing of the work on theories of the origin of vitamin A₂ is discussed by Collins, Love & Morton (1953b).

SUMMARY

1. The lipids from two species of newt (*Triturus cristata* and *T. carnifex*) have been examined by spectroscopic methods.

2. Vitamins A₁ and A₂ are both present as well as carotenoids, 7-dehydrosteroids and unidentified substances with selective absorption in the ultra-violet region.

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Studies in Vitamin A

25. VISUAL PIGMENTS IN TADPOLES AND ADULT FROGS

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Until recently vitamin A₂ was thought to occur only in fishes. The recognition by Wald (1942, 1946) of vitamin A₂ in amphibians has important consequences for the comparative biochemistry of vitamins A₁ and A₂ without which no assessment of possible evolutionary significance can be reached. The two previous papers in this series (Collins, Love & Morton, 1953a, b) described experiments bearing

on the problem. Wald (1946) discovered that tadpoles of *Rana catesbeiana* (the American bullfrog) contained vitamin A₂ only, and that it was replaced by vitamin A₁ in the adults. This observation prompted the present work which was undertaken to ascertain whether a similar state of affairs obtained in other species such as *R. temporaria* and *R. esculenta*.